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An investigation of the effects of plantain (*Plantago lanceolata*) ingestion on kidney function in sheep

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Georgina Ellen Lindsay

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Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Bachelor of Agricultural Science.

An investigation of the effects of plantain (Plantago lanceolata) ingestion on kidney function in sheep

by

Georgina Ellen Lindsay

The mechanism causing diuresis following ingestion of plantain as the sole dietary component of sheep was investigated. For three days two groups of sheep (n = 8) were fed either sufficient ryegrass or plantain (Agricom 'PG742') to provide a daily intake of 4 L of water per sheep. Water contents of feed and faeces and total urine volume were measured daily to determine water balance. Urine osmolality and total osmols excreted plus clearance values for creatinine and urea were calculated for each animal for each day. Following a ten day rest period, treatment groups were swapped and the trial was repeated. Plantain ingestion increased daily urine production by about 800 mL or 33% (P = 0.009) and reduced daily water balance by about 660 mL (P = 0.018) compared with the ryegrass treatment. Urine osmolality and specific gravity was significantly lower in plantain-fed sheep (P = 0.043) yet total excretion of solute was unaltered (P = 0.563). Creatinine and urea clearance values were the same for both feed treatments for 4 of the 6 days of the study period. These results confirm that ingestion of plantain causes a diuresis in sheep and the absence of effects on kidney clearance values suggests that the change in kidney function is likely to be confined to the distal convoluted tubules and collecting ducts. This new finding establishes the effect of dietary plantain on urine production in sheep as being a water diuresis.

Keywords: Plantain, *Plantago lanceolata,* water diuresis, urine, water balance, DCAD, packed cell volume, gloomerular filtration rate, clearance, vasopressin, antidiuretic hormone, vaptans

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Chapter 1

Introduction

Commonly known as plantains, Plantago is the genus of perennial herbaceous plants belonging to the Plantaginaceae family. Plantains typically have a prostrate growth pattern with a small number of the 200-odd species growing to a small shrub. Plantains have long oval leaves with vertically arranged veins and stems with compact, multi-flowered seed heads which emerge from the base of the plant. For centuries this Europe-originated plant has been recognised for its medicinal properties (Samuelsen, 2000; Tamura *et al.*, 2002) including prevention of stomach ulcers and bacterial and fungal infections. Other studies report plantain's ability to selectively disable cancerous cells (Galvez *et al.,* 2003) and notable antioxidant properties (Samuelsen, 2000).

The species used in agriculture, narrow-leaved plantain (*Plantago lanceolata*), is also thought to exhibit these medicinal properties (Stewart, 1996). One of these properties of agricultural and environmental importance may be the diuretic effect of plantain ingestion (Rumball *et al.,* 1997; Tamura *et al.,* 2002). Cáceres (1987) reported a 108% increase in urinary output in laboratory rats fed *Plantago major*, however only three reports have verified the potential of narrow-leaved plantain (*P.* lanceolata) to act as a diuretic in grazing ruminant animals (Deaker *et al.,* 1994; Wilman *et al.,* 1994; O'Connell *et al.,* 2016).

Despite scientific and anecdotal evidence of plantain-associated diuresis, there is minimal scientific literature explaining the mechanism of diuresis at a kidney function level. O'Connell *et al.*, (2016) reported that plantain causes increased production of more dilute urine yet total solute excretion is unchanged – suggesting plantain ingestion causes a water diuresis. However, there is no literature that describes the mechanism by which urinary output is increased. Understanding this may help isolate the compound or metabolite responsible, indicate long-term effects of grazing plantain on the animals and may hold environmental significance such as mitigating nutrient loading on the soil.

The diuretic potential of plantain is of interest to the wider agricultural community. Leaching of excess agricultural nutrients leading to diminishing water quality is a major environmental concern facing the sustainability of New Zealand's pastoral-based farming system. One potential mitigation technique is the use of diuretic plants to dilute animal urinary nitrogen in a larger water volume thus reducing nitrogen loading on the soil.

The aim of this project was to examine the physiological basis of plantain-induced diuresis in grazing ruminant animals. It has been shown that ingestion of plantain by sheep causes production of a

larger volume of more dilute urine, without altering total urinary osmol excretion (O'Connell *et al.,* 2016). From this it was hypothesised that plantain produced a water diuresis. The present study was conducted to confirm the presence of a water diuresis following consumption of plantain by sheep, using the model where plantain- and grass-consuming sheep received the same daily input of water in their diet, but was designed to extend this finding by collecting blood plasma samples to obtain measures of kidney clearance. Clearance values provide a measure of those aspects of kidney function that are separate from water transactions in the distal tubules and collecting ducts. The hypothesis under test was that plantain produces a diuresis by a process that does not involve alteration of glomerular and proximal tuble function, i.e. there would be a diuresis without change in clearance values. If supported by the results of this study, this hypothesis would provide additional strength for the previous hypothesis – that plantain produces a water diuresis in sheep.

Chapter 2

Literature Review

2.1 Plantain (*Plantago lancelata)*

With a long history as a minor forage species, narrow-leaved plantain (*Plantago lanceolata* L.) is becoming an integral part of temperate grazing livestock systems in New Zealand. Originally thought of as an unproductive weed, industry sources suggest over 5000 ha of plantain were sown in New Zealand in 2011 (Beef and Lamb NZ, 2002). This non-leguminous perennial herb is autumn-sown and often used as part of traditional ryegrass/white clover pasture mixes. Compared with uncultivated Plantago species, narrow-leaved plantain has been bred for increased productivity and a more upright growth pattern (Stewart, 1996). Genetic selection has led to improved tillering, larger leaves and improved summer growth, increasing its favourability as animal forage. One product of this development is Agricom's PG742 – a productive forage plantain cultivar with high tiller density and good cool season growth (Agricom Ltd., 2012).

2.2 Productivity

Reports on feeding value and growth rates of plantain are contrasting however it appears growth rates of livestock on plantain can match those on traditional pastures given correct plantain grazing management. Kemp *et al.* (2013) reported 80% and 66% increases in growth rates of lambs fed plantain/clover and plantain/chicory/clover mixtures, respectively, compared with perennial ryegrass/clover combinations. Beef and Lamb NZ (2002) reported variable animal performance results depending on severity of summer dry periods and residual herbage masses. In a Massey University grazing trial, carcass weight was 102 kg/ha greater on plantain-based pastures than ryegrass/white clover corresponding to a 33% increase in stocking rate (Kemp *et al.,* 2013). Liveweight gain has been shown to be limited by grazing below 5 – 6 cm or 1200 – 1500 kgDM/ha (Moorhead *et al.,* 2009; Beef and Lamb NZ, 2002; Kemp *et al.,* 2013); meaning grazing management of plantain is more critical than for hardier ryegrass-based pastures. Increased residuals up to 8 – 11 cm were found to increase lamb growth (Kemp *et al.,* 2013; Beef and Lamb NZ, 2002), however this greatly limited utilisation illustrating more careful pasture management requirements. Plantain can enhance lactational ability of the ewe with greater milk yield one week post-lambing and improved lamb liveweight at three weeks of age (Hutton *et al.,* 2011).

2.3 Mineral content

Compared with perennial ryegrass, plantain has elevated levels of many minerals (Stewart, 1996) however different cultivars have largely different mineral contents. Typically plantain has similar nitrogen content as ryegrass (Wilman *et al.,* 1993; Wilman *et al.,* 1994) and higher levels of calcium, copper, sodium and boron (Wilman *et al.,* 1993; Stewart, 1996; Hoskin *et al.,* 2006; Harrington *et al.,* 2006; Pirhofer-Walzl *et al.,* 2011) (see Appendix A).

2.4 Kidney function

The primary function of the kidneys, the major excretory organ of the body, is to maintain homeostasis of body fluid by regulation of blood composition. Microscopic functional units called nephrons regulate ionic composition, osmolarity, volume, pressure and pH of the blood plasma (Tortora *et al.,* 2000). Nephrons also control excretion of wastes and foreign substances through formation of urine. Urine production is a result of three processes – filtration, secretion and reabsorption.

Afferent arterioles divide to form a capillary network called the glomerulus. The pressure of blood in these glomerular capillaries provides a driving force causing water and other solutes to leave the blood by a specialised filtration process. This so-called 'glomerular filtrate' collects into the Bowman's capsule of the nephron from where it enters a single tube, a tubule, that passes largely through the inner region, the medulla, of the kidney and finally drains into a collecting duct which also receives the distal end of tubules from neighbouring nephrons (Tortora *et al.,* 2000). Within the tubules there is a large amount of activity by the cells lining their walls primarily directed at returning much of the glomerular filtrate back into the kidney interstitium and, thence, back to the body – reabsorption. Some substances, usually unwanted, can be added to the filtrate by these cells – secretion. Tubular reabsorption and secretion occur primarily in the proximal convoluted tubule of the nephron beyond which the filtrate flows through a small-diameter, specialised region of the tubule called the loop of Henle and thence into the distal convoluted tubule before reaching the collecting duct. Further processing of the filtrate (reabsorption and secretion), much of which is highly regulated, occurs in these latter regions and the resultant fluid is excreted from the ends of the collecting ducts as urine (Bray *et al.,* 1986).

2.4.1 Glomerular function

Glomerular filtration rate (GFR) is controlled by the relativity of pressures from the blood in the capillaries of the glomerulus and hydrostatic pressure exerted by the filtrate within the glomerulus. However the primary driver of GFR is blood pressure within the glomerular capillaries. Factors which increase glomerular blood pressure will cause an increase in GFR and, all other things being equal,

increase urine output. Likewise, factors which reduce GFR such as constriction of kidney arterioles during elevated sympathetic nerve activity, will reduce glomerular blood pressure, GFR and urine output.

2.4.2 Tubular function: reabsorption and secretion

Reabsorption of about 80% of the filtered fluid (i.e. water and solutes) takes place in the proximal convoluted tubules and accounts for the major energy expenditure of the kidneys (Tortora *et al.*, 2000). Primarily this function is directed at the reabsorption of sodium ions (Na^+) – the major cation of all extracellular body fluids. The major anion of body fluid, chloride (Cl^-) probably moves passively by electrostatic attraction to Na^+, and the return of these two ions causes water reabsorption – by osmosis. This primary function occurs spontaneously, i.e. it does not appear to be regulated and hence is termed – 'obligatory' reabsorption. The processes of reabsorption and secretion that take place in the other regions of the nephrons do appear to be regulated, hence the term – 'facultative' reabsorption to describe the component of reabsorption that is under homeostatic control.
Tubular secretion involves the movement of additional material into the tubular filtrate and is important in elimination of wastes, toxins and foreign compounds from the body fluids. It is also involved in excretion of potassium (K^+) ions and in renal regulation of acid-base balance.

2.5 Body water

Water constitutes approximately 60% of ruminant body mass (Akers *et al.,* 2008) 10-30% of which is stored in the rumen (Hecker *et al.,* 1964). Many chemical reactions such as energy synthesis and nerve impulse conduction require solution of constituents in water. 55-65% of this body water is contained within cells in the intracellular fluid (ICF) whilst the remaining 35-45% of body water is extracellular fluid (ECF) (Verbalis, 2003). ECF can be further categorised as fluid which surrounds the cells, interstitial fluid (ISF), and fluid within the blood, intravascular fluid (IVF) – better known as 'plasma'. Water flows between the IVF and the ISF, allowing transfer of nutrients and ions.

2.6 Water balance

Animals consume food and water to supply their bodies with nutrients and fluids. Along with excretion of wastes via urine and faeces, body water is also lost via body heat dissipation. Water loss is essential in normal body function, however excessive water loss causes dehydration which can lead to illness or death. Thus an innate regulation system of water movement is required.
The net difference between water intake and excretion is termed water balance, and is largely controlled via feedback mechanisms which include the juxtaglomerular apparatus (JGA) of the kidney and the hypothalamus of the brain (Campbell *et al.,* 2005). These feedback signals monitor and

adjust body solute concentrations to stabilise volumes and osmotic levels of body fluids and to maintain arterial blood pressure within the normal range (Verbalis, 2003).

2.7 Dehydration

If an animal undergoes sustained negative water balance, their body can become dehydrated. The rumen acts as a water reservoir and initially plasma volume is maintained by drawing water into circulation from the rumen (Cockram, 2014). However after four days under restricted water ingestion, water may be lost from the blood (IVF) causing an increase in plasma osmolality (Kataria *et al.*, 2007). At this point sheep may begin to show signs of dehydration which can include loss of body weight, increased percentage of blood which is composed of red blood cells (packed cell volume – PCV) or increases in plasma urea, plasma total protein or osmolality (MacFarlane *et al.,* 1961).

2.8 Osmolality (solute balance)

In addition to regulating the total balance of water intake and excretion (i.e. fluid volumes), the kidneys also regulate the osmolality of body fluids. Osmolality is effectively a measure of solute density (solute per unit volume). The passive osmotic movement of water from regions of low to high solute concentrations allows water excretion to be balanced by active and passive movement of solutes within the capillaries and filtrate of the nephron. Given sodium ions comprise greater than 90% of all cations in ECF, making it the main cation of body fluid overall, the primary function of the kidneys is to balance the intake and excretion of sodium with that of water.

The two major components of kidney function; regulation of Na^+ concentration and osmolality of ECF, are largely interrelated as changes in water turnover will induce concentrating/diluting effects on osmolality of ECF. For example during periods of dehydration, water loss occurs at proportionately greater rate than loss of solutes thus plasma osmolality increases.

Under conditions of elevated plasma osmolality, vasopressin (see Section 2.9) is secreted from the posterior pituitary gland of the hypothalamus to increase reabsorption of water in the kidneys and thus dilute body fluids. Simultaneously, aldosterone secretion (see Section 2.10) is supressed, thereby reducing reabsorption of Na^+ along with that of Cl^-; the net effect being production of urine with a lower volume but greater osmolality (Berkeley University, n.d.). Conversely, low osmolality (or excess body water) is countered by negative feedback signalling from the JGA to the hypothalamus, mainly via hormones aldosterone and angiotensin-II.

Urine specific gravity (USG) is linearly proportional to urine osmolality and indicates the ability of the kidney to concentrate or dilute urine over that of plasma (Parrah *et al.,* 2013). For sheep, the normal USG range is 1.015 – 1.045 (Swenson *et al.,* 1993).

2.9 Vasopressin

Vasopressin, also known as antidiuretic hormone, is the body's natural water conservation hormone. Secreted from the posterior pituitary gland by neurons in the hypothalamus (Campbell *et al.*, 2005) vasopressin binds to receptors on the renal distal tubules and collecting ducts of the kidney. Here it generates 'water channels' which facilitate reabsorption of solute-free water from the filtrate back into the blood stream (Tortora *et al.*, 2000). Simultaneously, vasopressin stimulates thirst which encourages the animal to ingest more water. Without vasopressin, the distal tubules and collecting duct are virtually impermeable to water resulting in a greater rate of urine production (see Section 2.11.2).

Vasopressin secretion is stimulated by two main homeostatic cues: elevated plasma osmolality (as identified in the hypothalamus) and reduction in blood pressure (identified in stretch receptors of the aorta). Simultaneously, signalling from the juxtaglomerular apparatus (JGA) causes increased reabsorption of Na^+ (and Cl^-) which also stimulates water reabsorption. Conversely, vasopressin secretion is inhibited if stretch receptors in the atria recognise elevation in blood volume – in attempt to rid the body of excess fluid.

2.10 Aldosterone

Aldosterone, a steroidal hormone which is secreted from the adrenal cortex, stimulates sodium reabsorption in the distal tubules. As for vasopressin, aldosterone secretion is increased during periods of elevated plasma osmolality or reduced blood pressure. If the adrenal cortex recognises elevated plasma osmolality, aldosterone secretion is inhibited thus less sodium is reabsorbed into the plasma. Reduction in blood pressure, recognised by JGA, stimulates a hormonal cascade called the renin-angiotensin-aldosterone system (RAAS) involving the ultimate secretion of aldosterone and hence stimulation of sodium reabsorption in the kidney (Tortora *et al.,* 2000).

2.11 Diuretics

Diuretics are substances that cause diuresis – increased production of urine (Tortara *et al.,* 2000). Diuretics interfere with normal maintenance of water balance by altering the kidney tubular environment or interfering with signal molecules, most commonly through inhibition of sodium reabsorption.

2.11.1 Osmotic diuresis

Osmotic diuresis occurs when more solute is presented to the tubules than their maximal reabsorption rate (Bray *et al.,* 1986). Osmotic diuretics are hydrophilic substances that increase osmotic concentration of the filtrate within the tubular fluid and hence block water reabsorption

(Atherton *et al.,* 1968). When such diuretics are present, water reabsorption is limited throughout the convoluted tubules (Mathisen *et al.,* 1981). Urinary flow rate is dependent upon urine solute content resulting in elevated urine osmolality and volume (Bray *et al.,* 1986).

Osmotic diuretics most commonly inhibit sodium ion reabsorption. For example, loop diuretics prevent reabsorption of sodium within the ascending limb of the loop of Henle by binding to the $Na^+/K^+/Cl^-$ co-transporter which inhibits reabsorption of calcium, potassium and sodium from the filtrate and thus draws water into the tubule (Wittner *et al.,* 1991; Tortora *et al.*, 2000).

Action of loop diuretics can be detected by elevated levels of calcium, potassium and sodium in the urine (Tortora *et al.*, 2000). Thiazide diuretics inhibit reabsorption of sodium in the distal convoluted tubule of the nephron by interfering with sodium-chloride transporters (Rose, 1991). This also causes water retention within the tubule which increases urinary output. Thiazide diuretics promote increased calcium reabsorption, hence are termed calcium-sparing diuretics. Elevated urinary potassium and low urinary calcium levels are characteristic of urine produced due to presence of thiazide diuretics.

Diuretics that inhibit reabsorption of sodium increase the amount of water and solutes which reach the more distal parts of the nephron. The distal convoluted tubules act to increase solute absorption but often cannot compensate for diminished potassium reabsorption leading to excess potassium excretion in the urine. Potassium-sparing diuretics act in the distal tubule by blocking sodium channels or acting as aldosterone antagonists to prevent sodium reabsorption (Horisberger *et al.*, 1987) and promote diuresis without excess loss of potassium.

The enzyme, carbonic anhydrase, is located in the proximal convoluted tubule of the nephron, where the greatest proportion (up to 55%) of urinary sodium is reabsorbed (Rose, 1991). This reabsorption process involves the exchange of sodium ions for hydrogen ions. Carbonic anhydrase inhibitors (CAIs) prevent this reabsorption process leading to accumulation of sodium in the tubule lumen which, in turn, inhibits water reabsorption and produces greater urine volume (Rose, 1991). Urine produced in a CAI-induced diuresis contains high levels of bicarbonate, therefore can often be characterised by elevated urinary pH (Rose, 1991). Typically CAIs are less effective diuretics as they affect only the proximal tubule and excess water here can be reabsorbed by more distal regions of the nephron (Rose, 1991).

2.11.2 Water diuresis and vaptans

A water diuresis is characterised by elevated production of dilute urine. Inhibition of vasopressin release and/or blockade of its receptors results in relatively impermeable collecting ducts and therefore elevated water loss without solute (Atherton *et al.,* 1968; Bray *et al.,* 1986). Unlike for osmotic diuresis, there is an inverse relationship between urine flow rate and urine osmolality, thus the amount of solute excreted is relatively constant (Bray *et al.,* 1986).

Vasopressin receptor antagonists, or vaptans, are a class of drugs used for treatment of water retention in the body (O'Connell *et al.*, 2016). Vaptans work by interacting with vasopressin receptors throughout the body. Unlike osmotic diuretics, vaptans encourage water loss but enable retention of solutes (Aditya *et al.*, 2014).

2.12 Plantain diuresis

Ingestion of plantain increases daily urinary volume (Wilman *et al.,* 1994; O'Connell *et al.*, 2016). O'Connell *et al.* (2016) found that ingestion of plantain caused production of a large volume of dilute urine indicative of a water diuresis. Sheep ingesting plantain produced 1.7 litres more urine than those fed grass on Day 1 of the trial and continued to produce about 500 mL more urine each day for the following 5 days of experimentation (O'Connell *et al.,* 2016). Urine specific gravity and osmolality measures showed plantain-fed sheep produced more dilute urine yet total solute excretion was unaltered by diet (O'Connell *et al.,* 2015). The mechanism causing this diuresis is unknown.

2.13 Clearance

Renal plasma clearance, known as clearance, is a measure of the volume of blood 'cleaned' or 'cleared' of a substance per unit time (mL/min) (Tortora *et al.*, 2000). A high clearance value indicates efficient excretion of a particular substance in the urine and is an important indicator of kidney function. Rate of clearance is dependent upon glomerular filtration, tubular reabsorption and tubular secretion. Clearance of substances which are neither secreted nor reabsorbed in the tubules gives a good indication of glomerular filtration rate (GFR). Such substances include creatinine, a waste product of creatinine phosphate catabolism in skeletal muscle, which is only secreted in very small amounts in the kidney tubules (Tortora *et al.*, 2000). Urea is both secreted and reabsorbed in the kidney tubules and hence is less illustrative of GFR.

2.14 Agricultural application

Leaching of excess agricultural nutrients leading to diminishing water quality is a major environmental concern facing the sustainability of New Zealand's pastoral-based farming system. The imbalance between nitrogen fertiliser applications, nitrogen use efficiency of pasture species and nitrogen requirements of grazing animals generates an oversaturation of nitrogen (N) in the pasture. Stock urine patches are the major contributor in a pastoral system with 60-90% of N consumed by dairy cows being returned to the soil in urine patches of up to 1000 kgN/ha, and 350-500 kgN/ha from sheep (Di *et al.,* 2007). Leaching of nutrients through the profile can contaminate nearby fresh water stores. Elevated nitrate concentrations in drinking water have been linked with methaemoglobinaemia, stomach cancer and childhood diabetes and in surface water can cause eutrophication, excess plant growth, algal blooms and the resultant decline in aquatic life. Growing

concern over these environmental impacts has led the industry toward innovative techniques to limit nitrate leaching. One potential mitigation technique is the use of diuretic plants in forages to dilute animal urinary nitrogen levels.

Chapter 3

Methods

3.1 Trial Design

3.1.1 Sheep and feeding regime

This study used 16 Romney-composite five-month-old ewe hoggets owned by commercial seed company PGG Wrightson Seeds (Hornby, New Zealand). In the period leading up to the trial, sheep were grazed on a mix of modern diploid perennial ryegrasses, approximately 2 years old, near Lincoln, Canterbury, New Zealand (Kimihia Research Centre). On 25 April 2016 they were transported 5.2 km to Lincoln University's Johnstone Memorial Laboratory (-43.644175 E, 172.451685 S) where they were held indoors for four days in individual metabolism crates with no access to drinking water. During the first night (25 April, Day 0) the animals were fed 4 kg fresh weight of perennial ryegrass (*Lolium perenne).* The following morning any feed refusals, urine and faecal matter were discarded and the animals were allocated to one of the two dietary treatment groups; plantain (*Plantago lanceolata*, cultivar PG742, n = 8) or ryegrass (*Lolium perenne*, diploid, n = 8) (Day 1). The sheep were held in two separate rooms with five animals per treatment in one room and three animals per treatment in the other.

Feed, supplied by PGG Wrightson Seeds from the Kimihia Research Centre, was allocated in quantities equating to four litres of daily feed water (4.5 – 5.2 kg fresh weight) per head of sheep. Fresh weight allocations of the feed were calculated based on the dry matter content of a sample of the previous day's feed. Any refused feed was weighed and discarded, and this weight subtracted from the weight of feed offered to give each animal's feed intake for that day.

All procedures in this trial were conducted under approval of the Lincoln University Animal Ethics Committee.

3.1.2 Liveweight

Liveweight was measured immediately prior to and after the trial using electronic scales (XR5000, Tru-test, Auckland, New Zealand). Daily weight gains of each sheep were calculated using Day 4 weight less Day 0 weight divided by 4.

3.1.3 Feed harvesting

Plantain and ryegrass monocultures were grown in separate paddocks at the Kimihia Research Centre. Each was harvested daily at about 0830 h using a motorised forage harvester (Wintersteiger Cibus F, Wintersteiger AG, 4910 Ried, Austria) which weighed and collected required pasture into

synthetic wool fadges. Both pasture types were approximately two years old with pre-harvest pasture masses of about 1200 kgDM/ha to maximise leaf to stem ratio and thus ensure high pasture quality. Feed was transported immediately to the Johnstone Memorial Laboratory where it was weighed into aliquots that were placed in each individual sheep's feed bin within half an hour of being harvested. One fresh sample of each feed type was collected and frozen each day. Three samples of each feed were collected daily and weighed into perforated bags. They were then placed in a 90°C oven for 24 hours or until dry, before being reweighed to calculate dry matter percentage of each day's feed: average dry weight /average fresh weight × 100.

3.1.4 Urine and faeces collection

Urine and faecal matter was separated using collection trays and buckets under each metabolism crate. To prevent cross-contamination, net separators (shade cloth stretched over a frame and positioned on top of the collection buckets) were used to trap faeces and allow urine to flow into the collection buckets.

The metabolism crates were cleaned daily and faeces collected and weighed for each animal. One fresh faeces sample per animal was collected in a small labelled snaplock plastic bag to be freeze-dried for subsequent analysis. Two other faeces samples per animal were collected in perforated bags and weighed for oven drying at 90°C for 48 hours (an additional 48 hour drying period was allowed for larger samples). Once dry, the samples were reweighed and dry matter percentages were calculated for each sample (i.e. dry weight/fresh weight × 100). The average of the two samples for each animal was used to determine faecal water content.

To prevent nitrogenous losses via volatilisation, urine was acidifed with 250 mL of 5% sulphuric acid that had been placed in the collection trays prior to the start of each day's collection period. Total daily urine volume of the collection tray was measured at about 0900 h using a volumetric flask. A 50-70 mL aliquot of the acidified urine sample was collected in a polycarbonate specimen container and stored in a freezer (-20 °C). Collection trays were rinsed with tap water before being returned to each crate until a non-acidified (fresh) urine sample (50-70 mL) could be collected. Following collection of the fresh sample, acid was added to the collection trays for the remainder of the 24-hour collection period. Total daily urine volume was determined as: total volume of acidified urine in the collection tray + volume of fresh sample – volume (250 mL) of 5% sulphuric acid.

3.1.5 Water balance

Daily water balance was determined for each animal by subtracting the total urine volume plus faecal water volume from the total water content of the feed eaten.

3.1.6 Blood collection

A 10 mL venous blood sample was obtained by jugular venepuncture with manual restraint from each animal at approximately 1000 h each day using a 0.9 × 25mm needle (Becton Dickinson Vacutainer Systems PrecisionGlide, New Jersey, USA) with evacuated plastic tubes containing lithium ethylenediamine tetraacetic acid as anticoagulant (Becton Dickinson Vacutainer Systems, New Jersey, USA). Immediately following removal from the vein, the tubes were gently inverted a few times to ensure dispersal of the anticoagulant.

Packed cell volume was determined in duplicate using glass microhaematocrit tubes (Becton Dickinson Vacutainer Systems, New Jersey, USA) plugged with vinyl plastic putty (Leica Critoseal, Wetzlar, New York, USA). The tubes were spun in a microhaematocrit centrifuge (Heraeus Sepatech Haemofuge, Hanau, Germany) for 4 minutes before measurement using a microhaematocrit reader (Hawksey Micro-haematocrit Reader, London, England). The average of the two readings was recorded as the packed cell volume (PCV) for each animal's blood sample.

Blood samples were centrifuged in their collection tubules at approximately 1000 g for 20 minutes to separate plasma from the cells and two 1-2 mL aliquots of plasma were transferred to polythene cryovials for storage in a freezer at -20 °C.

3.1.7 Urine pH and specific gravity

Within 2 hours of collection, urine pH was determined for the fresh urine samples using a portable pH reader (Horiba Laquatwin compact pH meter pH33, Shiga, Japan). Specific gravity was determined using a portable refractometer (GrandIndex, HongKong, China). The remainder of the fresh urine samples in their collection containers were packed into a plastic snaplock bag and placed in a freezer (-20 °C) for storage.

3.2 Plasma and urine analysis

Analysis of plasma and urine was performed at a commercial analytical laboratory (Gribbles Veterinary, Christchurch, New Zealand) using an automated analyser (Hitachi Modular P800, Roche Diagnostics Corporation, Indianapolis, IN, United States of America) with kits supplied by Roche Diagnostics Corporation for sodium (plasma and urine), urea (plasma), creatinine (plasma and urine), total protein (plasma) and micro protein (urine). Sodium was analysed with an ion-selective electrode. Urea in plasma was analysed by measuring the decrease in ultraviolet light absorbance due to consumption of reduced nicotine adenine dinucleotide (NADH) following hydrolysis of urea by urease. Creatinine was measured following its enzymatic conversion to sarcosine and determination of the amount of hydrogen peroxide emitted using a Trinder's reaction acceptor which produces a red benzoquinone-imine with high light absorbance. Total protein in plasma was measured using the Biuret reaction which forms a coloured complex with copper ions and the micro protein method for

urine measured the turbidity following denaturation of protein in alkaline conditions and addition of benzethonium chloride. Urinary urea and ammonia concentrations were measured by the Analytical Services Unit at Lincoln University on an automated analyser (Randox RX Daytona Analyser, Randox Laboratories, Crumlin, County Antrim, United Kingdom) using enzymatic kits supplied by the manufacturer. Urea in urine was measured as described above for plasma, and ammonia was assayed by measuring ultraviolet light absorbance following combination of ammonia with α-ketoglutarate and reduced nicotine adenine dinucleotide phosphate (NADPH) by the enzyme glutamate dehydrogenase. Urinary nitrogen concentration was also determined at Lincoln University Analytical Services on an automated analyser (Variomax CN Analyser, Elementar Analysensystem GmbH, Hanau, Germany) using the Dumas combustion method.

Protein, creatinine, sodium, ammonia, urea and nitrogen measurements were taken using acidified urine samples. These were adjusted to account for the sulphuric acid volume (250 mL) using the following formula:

$$Adjusted\ value = Acidified\ value\ x\ \frac{urine\ volume\ (mL) + 250\ mL}{urine\ volume\ (mL)}$$

3.2.1 Osmolality

Specific gravity (SG) readings obtained from the refractometer were converted to osmolality values using the formula: $urine\ osmolality\ (mosmols/kg)\ =\ (SG\ -\ 1.000)\ X\ 32000.$
This is based on the relationship between these 2 variables being linear and that a urinary specific gravity of 1.010 corresponds with an osmolality of 320 mosmols/kg. The urine osmolality value was multiplied by the total daily urine volume for each sheep for each day to give the total number of omsols excreted per animal per day.

3.2.2 Clearance calculations

Plasma and urine concentrations of creatinine and urea were used with daily urine volumes to determine clearance values using the following formula:

$$Clearance\ \left(\frac{mL}{\min}\right) = \frac{Daily\ urine\ volume(mL)}{60\ x\ 24} x\ \frac{Urine\ concentration\ \left(\frac{\mu mol}{L}\right)}{Plasma\ concentration\ (\frac{\mu mol}{L})}$$

3.3 Feed composition analysis

Analysis of feed samples was performed at a commercial analytical laboratory (Hill Laboratories, Hamilton, New Zealand). Dry Organic Matter Digestibility (DOMD) and 1-12 AFRC were used to calculate Metabolisable Energy (ME) content of the feeds using the Lincoln University standard

formulae. Sodium content was determined via nitric acid/hydrogen peroxide digestion followed by ICP-OES. DCAD – dietary cation-anion difference was calculated based on the dietary concentration of cation minerals sodium (Na) and potassium (K) minus that of the anion minerals chloride (Cl) and sulphur (S). The chloride measurement for plantain in Day 3 of Week 1 (0.89%) was discarded as it was significantly lower than expected and generated an outlandish DCAD value for that day (603 mEq/kgDM). This value was replaced by the average chloride value for all other plantain measurements (2.66%) to generate a more realistic DCAD value (115 mEq/kgDM). Feed nitrogen content was determined using the Dumas combustion method.

3.4 Statistical analysis

For each variable, data for each animal over all 3 days (n = 3) were averaged as determined by a pivot table using Microsoft Excel 2010. The effect of grass and plantain feed treatments were then analysed via the Student's Paired *t*-test using computing software Minitab 16. Due to the apparent difference between Week 1 and Week 2 results, significant data were then analysed on a per day basis for each measurement using the Student's Two-sample *t*-test.

Feed compositional data were analysed using a Student's two-sample *t*-test. Feed dry matter % (DM) measurements were averaged for each day (n = 3) and daily sodium, metabolisable energy (ME), DCAD and nitrogen measures were used to compare feed treatments with each week analysed separately.

All results were considered significant if $P < 0.05$.

Chapter 4

Results

Table 1: Grand means (± S.E.M.) for daily measurements from a study of sheep consuming ryegrass or plantain diets for 3 days in each of 2 weeks.

	Ryegrass (n = 8)	Plantain (n = 8)	Significance (*P* value)
Water transactions			
Water ingested (mL)	3835.4 ± 52.7	3972.2 ± 9.3	0.017
Urine volume (mL)	2507 ± 156	3326 ± 149	0.009
Fresh faecal weight (g)	709.1 ± 33.8	685.1 ± 25.9	0.531
Faecal water (mL)	506.9 ± 26.9	485.8 ± 22.7	0.472
Water balance (mL)	821 ± 130	160 ± 142	0.018
Urine components			
pH	8.53 ± 0.08	8.47 ± 0.07	0.641
Ammonium (mmol/L)	16.52 ± 2.72	8.06 ± 1.94	0.026
Nitrogen (g/L)	4.97 ± 0.33	2.98 ± 0.10	< 0.001
Daily urine nitrogen (g)	114.8 ± 3.4	97.5 ± 4.6	0.005
Protein (g/L)	0.12 ± 0.01	0.08 ± 0.01	0.007
Creatinine (umol/L)	3597 ± 245	2277 ± 111	< 0.001
Urea (mmol/L)	116.64 ± 7.72	75.61 ± 2.77	< 0.001
Sodium (mmol/L)	41.30 ± 2.62	76.53 ± 5.09	< 0.001
Urine Specific Gravity (no units)	1.014±0.001	1.011±0.001	0.043
Osmolality (mosmol/kg)	456.7 ± 28.7	353.9 ± 29.1	0.043
Total osmol excretion (mosmols/day)	1086.7 ± 76.1	1157.3 ± 85.5	0.563
Plasma components			
Packed cell volume (%)	38.208 ± 0.5	38.729 ± 0.566	0.351
Total protein (g/L)	72.625 ± 0.999	72.708 ± 0.989	0.839
Creatinine (umol/L)	58.42 ± 0.81	61.33 ± 1.33	0.013
Urea (mmol/L)	5.57 ± 0.20	6.196 ± 0.224	0.027
Sodium (mmol/L)	145.250 ± 0.327	146.042 ± 0.284	0.035
Kidney function			
Creatinine clearance (mL/min)	98.71 ± 2.95	83.41 ± 2.96	0.002
Urea clearance (mL/min)	38.37 ± 1.90	30.73 ± 1.60	0.002

3.1 Water transactions

Total water ingested in the feed was essentially similar (about 4 L) for both diets (Figure 1); the significantly lower mean value for Ryegrass sheep (Table 1) being largely attributable to an uncharacteristic reduction (P = 0.034) in these animals on Day 2 of Week 2 (Figure 1).

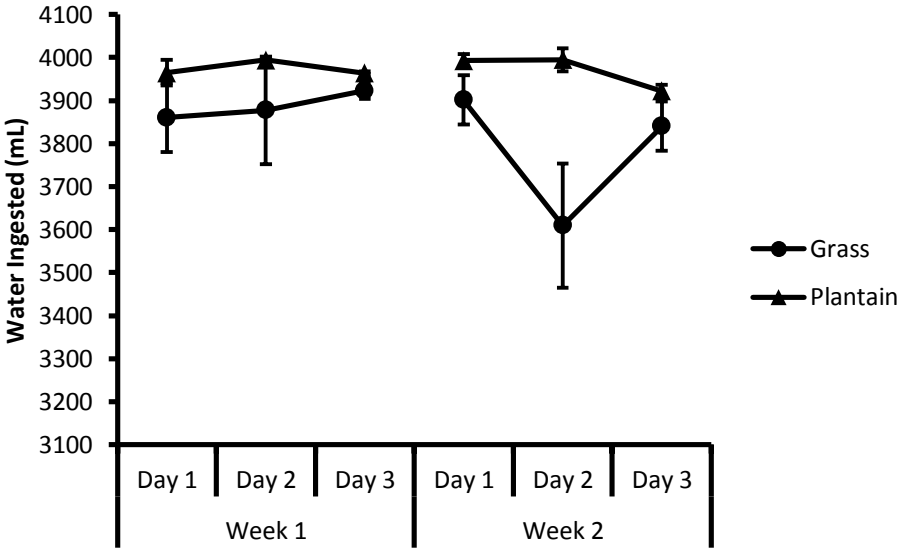

Figure 1: Mean daily water intake of sheep consuming ryegrass (Grass) or plantain (Plantain) diets for 3 days in each of 2 weeks. Vertical bars show ± S.E.M.

Urine volumes were significantly affected by feed type. Sheep ingesting plantain produced on average 819 mL more urine per day than those fed ryegrass (Table 1 and Figure 2).

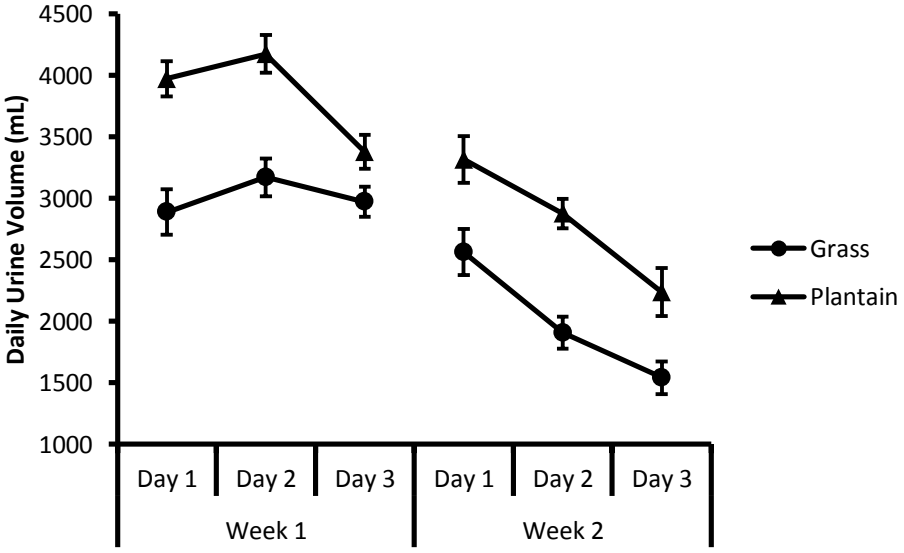

Figure 2: Mean daily urine volumes of sheep consuming ryegrass (Grass) or plantain (Plantain) diets for 3 days in each of 2 weeks. Vertical bars show ± S.E.M.

Fresh faecal weight and faecal water tended to be higher in Ryegrass sheep than in Plantain sheep, but neither were significant (Table 1).

Throughout the study period, water balance was consistently lower in the Plantain sheep (Table 1, Figure 3).

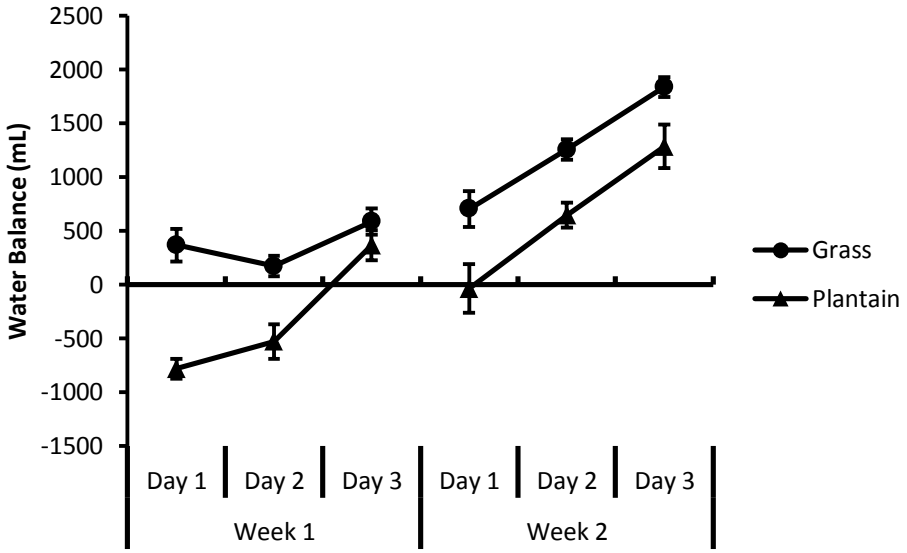

Figure 3: Mean daily water balance of sheep consuming ryegrass (Grass) or plantain (Plantain) diets for 3 days in each of 2 weeks. Vertical bars show ± S.E.M.

3.2 Urine components

There was no effect of dietary treatment on urinary pH (Table 1); however sheep fed plantain had lower urinary concentrations of nitrogen, protein, ammonium, creatinine and urea than the sheep on ryegrass (Table 1). In contrast, plantain-fed sheep had significantly greater urine sodium concentrations than sheep fed ryegrass (Table 1).

Also, overall, plantain-fed sheep produced urine with a lower specific gravity which translates as lower osmolality than the sheep fed ryegrass (Table 1), however the total number of osmols excreted per day did not differ between the dietary treatments (Table 1).

3.3 Plasma components

There was no difference in packed cell volume or total protein concentration of plasma between plantain-fed and grass-fed sheep (Table 1). Plantain-fed sheep had higher plasma creatinine, urea and sodium concentrations than the ryegrass-fed sheep (Table 1).

3.4 Kidney function

Overall creatinine and urea clearances were lower in plantain sheep than in the grass sheep (Table 1). However, this finding derives from Days 2 and 3 of Week 2, being the only occasions when significant differences occurred (Figures 4 and 5).

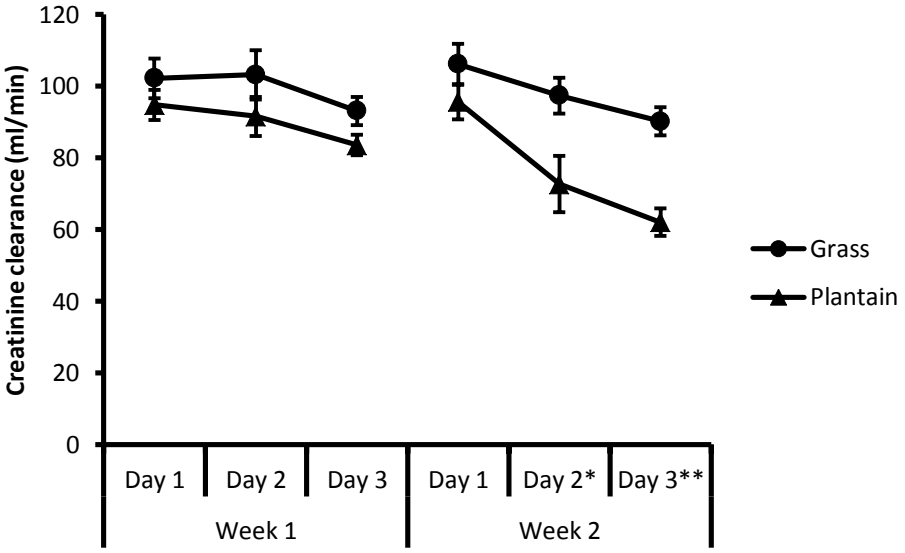

Figure 4: Mean creatinine clearance rates (ml/min) of sheep consuming ryegrass (Grass) or plantain (Plantain) diets for 3 days in each of 2 weeks. Vertical bars show ± S.E.M. * (*P* < 0.05), ** (*P* < 0.001).

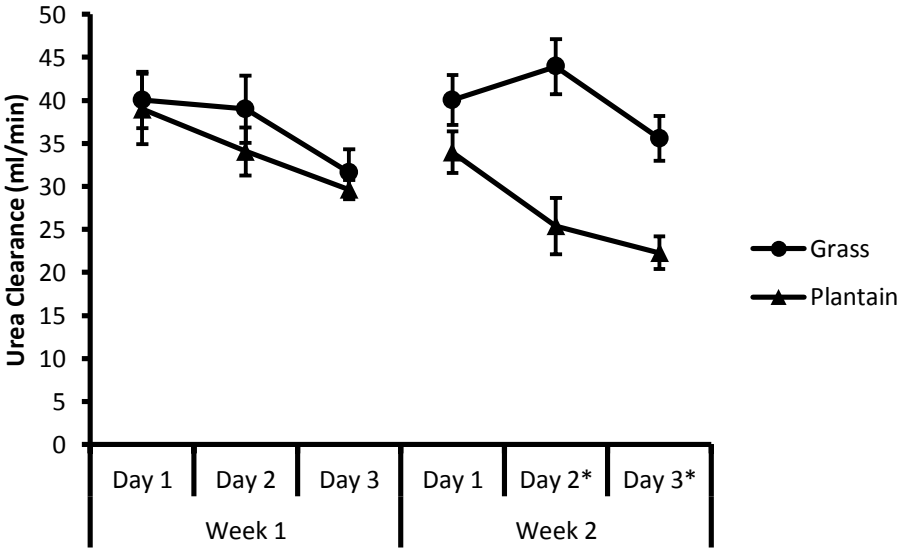

Figure 5: Mean urea clearance rates (ml/min) of sheep consuming ryegrass (Grass) or plantain (Plantain) diets for 3 days in each of 2 weeks. Vertical bars show ± S.E.M. * (*P* < 0.05).

3.5 Feed composition

The two diets were fairly well matched for ME content, although this was slightly higher in plantain than in ryegrass in Week 1 (Table 2). Feed DM % was lower in plantain in both weeks but only by a

few percentage units (Table 2). Both feeds had equal nitrogen contents for both weeks (Table 2). Plantain had a higher sodium concentration than ryegrass (Table 2). DCAD values of the two diets were similar in Week 1 but lower in plantain in Week 2 (Table 2).

Table 2: Partial compositional analysis of the diets fed indoors once-daily to sheep for 3 days in each of 2 separate weeks. Data are mean (± S.E.M.) for the 3 days of each week.

		Grass (n = 3)	Plantain (n = 3)	Significance (P value)
Dry matter (DM) %	Week 1	17.8 ± 0.90	13.4 ± 0.55	0.025
	Week 2	17.5 ± 0.24	16.5 ± 0.097	0.057
Metabolisable energy (MJ/kgDM)	Week 1	11.7 ± 0.15	12.23 ± 0.15	0.06
	Week 2	11.5 ± 0.15	11.6 ± 0.058	0.603
Nitrogen (mg/g)	Week 1	2.33 ± 0.03	2.37 ± 0.07	0.698
	Week 2	2.37 ± 0.13	1.97 ± 0.03	0.101
Sodium (g/kgDM)	Week 1	3.43 ± 0.09	7.57 ± 0.20	0.003
	Week 2	1.93 ± 0.73	7.16 ± 0.37	0.024
DCAD (mEq/kgDM)	Week 1	234 ± 33	130 ± 33	0.114
	Week 2	208 ± 27	99 ± 18	0.044

3.6 Liveweight

Both groups of sheep lost weight during the study period, but this was greatest in the Plantain sheep (Table 3).

Table 3: Mean (± S.E.M.) daily loss of liveweight of sheep consuming ryegrass or plantain diets for 3 days in each of 2 weeks.

Liveweight loss (kg/day)	Grass (n = 8)	Plantain (n = 8)	Significance (P value)
Week 1	0.9 ± 0.13	1.2 ± 0.12	0.079
Week 2	0.8 ± 0.13	1.2 ± 0.10	0.053

Chapter 5

Discussion

Ingestion of plantain caused a water diuresis – production of larger volumes of more dilute urine however total excretion of solutes was unchanged. Creatinine and urea clearance data indicated no impact on kidney glomerular function for 4 of the 6 days of experimentation which establishes the case that plantain affects reabsorption of water in the renal tubules and collecting ducts of the kidney.

5.1 Confirmed water diuresis

Compared with those fed grass, plantain-fed sheep consistently produced larger volumes of dilute urine without altering total solute excretion – indicative of a water diuresis. Unlike osmotic diuresis whereby urine flow rate is proportional to urine concentration (Bray *et al.,* 1986), plantain caused loss of water without excess solute excretion. Sheep fed plantain produced on average 33% (or 819 mL) more urine per day than those fed grass. However, specific gravity and osmolality measurements indicate a 77% reduction in urine solute concentration of Plantain sheep when compared with their grass-fed counterparts. Production of a greater volume of more dilute urine meant total excretion of solutes did not differ between diets. This diuretic effect was also reported by O'Connell *et al.* (2016) whereby plantain ingestion increased daily urine excretion and decreased urine concentration but did not affect total osmolar excretion.

5.1.1 Voluntary feed intake

Voluntary feed intake of individual animals was a potential source of error contributing to greater water ingestion by plantain-fed sheep. Total water ingested by treatment groups was largely the same for both diets – the significantly lower water intake of Ryegrass sheep on Day 2 of Week 2 being largely attributable to reduced feed intake of two individuals. From a purely observational basis, throughout the trial there was often more grass left behind by the sheep whereas the plantain was usually all consumed. Removal of the two outlier sheep on Day 2 of Week 2 would have increased the average water ingestion of the grass-fed group by over 200 mL, potentially eliminating the significant difference in water ingestion between feed treatment groups. Although the mean water ingested overall per sheep per day was significantly greater in plantain-fed sheep, the difference was confined to only 1 of the 6 days of the trial, indicating that the equal allocation of water for each sheep was achieved well.

Calculating water balance removes any bias of voluntary feed intake by illustrating the magnitude of urine production relative to water ingestion. Water balance was consistently lower in plantain sheep than ryegrass sheep for each day of experimentation. Despite plantain-fed sheep ingesting 3.6% more water, they produced 33% more urine than their grass-fed counterparts.

5.1.2 Evaporative losses and feed DM allocation

A consequence of allocating feed based upon total water input is that Ryegrass sheep may have had greater evaporative losses due to feed intake – a potential source of error. It was assumed evaporative water losses were similar for animals in both treatments. Evaporative losses via respiration and insensible loss are most significantly affected by temperature and diet (Degen *et al.,* 1981). The effect of temperature was negated by housing the animals together; however the significantly greater feed DM content of grass in Week 1 meant grass-fed sheep were allocated more DM than those fed plantain. Degen *et al.* (1981) found that evaporative water losses increase with increased feed intake suggesting grass-fed sheep may have had greater insensible water losses. This is thought to be due to additional metabolic energy requirements for nutrient assimilation and a corresponding rise in body temperature and therefore water loss (Degen *et al.*, 1981). In future studies, feed could be offered on a dry matter basis and each animal drenched with corresponding amounts of water to equalise water inputs. This would eliminate any bias associated with different dry matter contents of feed.

5.1.3 Mineral content and solute balance

Elevated urine volume of plantain-fed sheep may be due to increased mineral loading in the feed. Ledgard *et al.* (2015) reported a correlation between higher mineral content of feed and an increase in urination frequency and urine excretion in lactating dairy cows. In the present study, urine from the grass-fed sheep had higher concentrations of all constituents measured excluding sodium which was almost twice as concentrated in Plantain sheep urine. In these sheep natriuresis (excretion of sodium in urine) was obligatory for their homeostasis given the much higher sodium content of plantain than that of grass. Plasma sodium content was held at normal levels by excreting the sodium load in the urine thus urinary sodium concentration of Plantain sheep was almost double that of Ryegrass sheep. Despite this, total urine osmolality was unaltered by diet confirming that other solutes in the grass (nitrogenous compounds, including ammonium, protein, creatinine and urea) must balance total solute excretion in the plantain-fed sheep. It is unlikely that feed mineral content is the cause of plantain-induced diuresis given equal total daily osmols excreted in the urine independent of feed type.

5.1.4 Weight loss

Over the duration of the experiment plantain and grass fed sheep lost 1.2 and 0.8-0.9 kg of liveweight per day, respectively. Given water loss can account for 45% of weight loss in sheep (MacFarlane *et al.,* 1963) it is likely that the weight loss is purely reflective of loss of body water. This is highly likely considering animal liveweights recovered following the ten day rest period before treatment reversal.

Alternatively, the weight loss may be due to mobilisation of body tissues. During periods of greater physiological demand body fat, and to a lesser extent muscle, is mobilised to meet energy demand (Geenty, 1983). Use of radioactive labels such as sodium thiosulphate can be used to detect changes in extracellular water (MacFarlane, 1975). Changes in concentration of such chemicals following ingestion of plantain could indicate whether ingestion of plantain causes water loss from the rumen and gut or extracellular compartments. Slaughtering the animals post-trial could be useful to detect changes in body composition. Alternatively, less invasive measures such as subcutaneous fat sample analysis for adipocyte size and tissue depth, blood tests for non-esterfied fatty acids (NEFA) (Reid *et al.,* 1986) or simply body condition scoring could be used to indicate changes in body composition. These changes in body tissues would influence water balance and thus are an important consideration for future research.

5.1.5 Dehydration

Neither treatment group showed any indication of dehydration. Despite losing a lot of fluid, packed cell volume (PCV) of blood was similar among treatment groups and did not exceed the normal range (27-45%) (Merck Sharp & Dohme Corporation, 2015). Similarly, plasma sodium and total protein was unaltered by diet, also indicating that the animals were not under water stress. This eliminates any effects of dehydration on animal health or performance associated with feeding plantain in the short-term however it would be interesting to determine if the animals do undergo water stress after prolonged periods of plantain-induced diuresis.

5.2 Unaltered kidney function

Creatinine and urea clearance values were unaltered by diet for four of the six days of experimentation. Based on the assumption that creatinine clearance is equal to GFR this suggests the effects of plantain ingestion must be confined to water transactions in the distal tubules and collecting duct.

This finding is limited by the assumption that plasma concentrations in spot blood samples used to calculate clearance rates are representative of urine produced over the whole 24-hour period.

Acidified urine samples used to analyse creatinine and urea concentrations were effectively collected over a 24-hour period starting and ending at 0900 h. The blood sample taken at 1000 h that day was used for creatinine and urea plasma concentrations and calculation of clearance. In order to more accurately assess GFR, catheters could be used to take simultaneous blood and urine samples from the jugular vein and urethra at successive intervals and used to calculate clearance (Maloiy *et al.,* 1969).This direct urine collection method would also eliminate evaporative losses from urine collection buckets which may have biased measurements of urine concentration.

5.2.1 Vasopressin release or vasopressin receptor antagonist (vaptan)

The fact that plantain induces a water diuresis without affecting GFR suggests the diuretic compound must act in the distal renal tubules and collecting duct of the kidney. It could be hypothesised that a compound or metabolite of plantain acts to limit production or release of vasopressin from the hypothalamus, or acts as a vaptan by blocking vasopressin receptors. Analysis of plasma vasopressin levels of plantain- and grass-fed sheep could confirm the former hypothesis.

5.3 Discrepancy between week 1 and week 2

Although the trial was replicated, the data collected during Week 2 were very different to those of Week 1. As expected, sheep fed plantain in Week 1 produced 1838 ± 54 mL greater urine volumes than when the same sheep were fed ryegrass the following week. However sheep fed ryegrass in Week 1 actually produced 200 ± 149 mL more urine per day than they did ingesting plantain in the following week ($P < 0.001$). Because urine volumes of both treatment groups were much lower in Week 2, it is likely that feed type did not have carry over effects on the following week's urine production. It is more likely that data in Week 2 are very different due to some change within the plantain forage.

Interestingly, from Week 1 to Week 2 the change in DM content of grass was almost negligible (0.258%) yet plantain dry matter content increased by over 3% (Table 3). Plantain in Week 2 also had reduced ME, nitrogen content and DCAD from that of Week 1. The fact that feed DM, ME, N and DCAD differed substantially in plantain but not in ryegrass suggests the mechanism for this change was reflective of a change in the plantain and not a climatic effect. A change in plantain in Week 2 may explain the discrepancy between data collected in Weeks 1 and 2 of experimentation including the significant reduction in clearance of plantain-fed sheep in the latter days of Week 2.

5.4 Urinary nitrogen

Urinary nitrogen (N) concentration was significantly lower in plantain-fed animals. Generally urinary N excretion is proportional to intake (Tas *et al.,* 2006; Higgs *et al.,* 2012), however the slight

difference in DM intake of treatment groups is unlikely to expain the 40% reduction in urine N concentration of sheep fed plantain compared with those fed ryegrass. Box *et al.* (2016) demonstrated a 56% reduction in urine N concentration of plantain-fed dairy cows. This suggests plantain ingestion reduces surplus metabolised N which could be attributable to excess gaseous N emissions, partitioning of excretory N into the faeces or protein protection against degradation in the rumen (Stewart, 1996).

The urinary nitrogen reducing properties of plantain have been widely reported within dairy cow literature (Woodward *et al.,* 2012; Totty *et al.,* 2013; Box *et al.,* 2016) yet there are minimal reports for N excretion in sheep grazing plantain. This is primarily due to greater stocking densities of dairy farms thus higher nitrogen loading on the soils. Irrespective of species, the reduction in urinary N content of ruminants grazing plantain is hugely important in an agricultural context. Any urinary compositional changes will affect the way in which the urine interacts with soil. Presuming sheep excrete 4 L of urine per m^2 (Williams *et al.,* 1994) at a concentration of 2.98 gN/L, N would be applied in sheep urine patches at 11.92 gN/m^2 or 119 kgN/ha (4 L/m^2 * 2.98 gN/L * 10,000 m^2/ha / 1000g/kg = 119 kgN/ha). This is comparably lower than grass-fed sheep excreting urine at 4.97 gN/L or 199 kgN/ha in a urine patch. Compared with other literature, these values for nitrogen loading of sheep urine patches is surprisingly low with most reports approximating 300-500 kgN/ha (Di *et al.,* 2007). Sheep urine N concentrations typically range from 1.4-17.8 gN/kg (Hoogendoorn *et al.,* 2010) which may explain the relatively low level of nitrogen calculated in these theorectical urine patches. Irrespective of exact figures, being a major contributor of oversaturation of nutrients in grazed pastoral systems, a reduction in nutrient concentration of stock urine patches could greatly reduce nitrate leaching (Di et al., 2007). Lysimeter studies revealed a proportionally greater reduction in N leaching with lower urine N rate (Ledgard *et al.,* 2015) which is also evident, theoretically, in the present trial. In grazed pasture systems the amount and fate of nitrogen is determined by the number of urination events, volume of urine produced and the concentration of nutrient in the urine (Hoogendoorn *et al.,* 2010). In order to fully understand the implications of these findings more information on urine frequency and volume is required.

5.5 Urine pH and feed DCAD

Urine pH was not altered by feed type. O'Connell *et al.* (2016) found feeding plantain (*cv.* 'Ceres Tonic', Agricom) reduced urinary pH by almost 2 units. Given this trial used a different cultivar of plantain ('PG742') which may differ to 'Tonic' in DCAD; this may be explained by the curvilinear relationship between feed DCAD and urine pH. Lean *et al.* (2005) showed DCAD has minimal impact on urinary pH until it reaches approximately 200 mEq/kgDM. The relatively low dietary DCAD values

of plantain used in this trial (99 – 234 mEq/kgDM) may explain why, despite previous reports of plantain-induced urine acidification, there was no evidence of such effects.

5.6 Further study

The amount of plantain required to induce the water diuresis is unknown. Dose-response trials could be run to determine the amount of plantain in the diet required to generate the diuresis. Although some pure plantain swards are sown, plantain is typically used in pasture mixes with ryegrass and white clover (Beef and Lamb NZ, 2002). These results could be used to determine the amount of plantain that needs to be available in the sward to gain the benefits of the diuresis.

The component of plantain responsible for the diuresis is also unknown. Plantain contains many putative diuretic compounds including iridoid compound aucubin which promotes removal of uric acid from tissues and its excretion (Stewart, 1996; Deaker *et al.,* 1994). Plantain also contains 0.7-5% catapol, a putative diuretic (Bowers *et al.,* 1992). Alternatively the compound responsible may be a metabolite of plantain produced during its digestion. Further research could isolate various compounds and test them individually for diuretic properties. This has potential application on-farm as the isolated compound could be applied to feed or administered to animals via drench or inoculation to cause diuresis.

The long-term effects of feeding plantain should be investigated including health and welfare implications of sustained diuresis. Excess excretion of minerals such as calcium may have adverse side-effects such as kidney stones or mineral deficiencies (James *et al.,* 1984). Deaker *et al.,* (1994) showed lambs grazing plantain rather than perennial ryegrass had larger kidneys at slaughter and attributed this to increased metabolic loading of plantain-fed sheep. A long-term trial could be conducted in attempts to demonstrate this kidney hypertrophy effect by slaughtering and weighing the kidneys of the sheep post-trial. This may also reveal impacts of amplification of kidney size and/or function.

5.7 Conclusions

- Feeding plantain as the sole dietary component for sheep causes a water diuresis – production of a larger volume of more dilute urine without altering total solute excretion. However the compound responsible and amount of plantain required to maintain this effect is unknown.

- The mechanism of this diuresis did not affect creatinine and urea clearance for four of the six days of analysis suggesting plantain does not impact glomerular and proximal tubule

function. These characteristics of the diuresis suggest a compound or metabolite of plantain may inhibit vasopressin or its renal receptors.

- Despite having a similar feed nitrogen and dry matter content as ryegrass, feeding plantain significantly reduced urinary nitrogen concentration and total nitrogen excreted confirming plantain's potential to help mitigate nitrogenous losses from grazed pastoral systems.

Appendix A

Plantain mineral content

Table 4: Macromineral content of plantain compared with ryegrass adapted from Wilman *et al.,* 1993, Wilman *et al.,* 1994 and Pirhofer-Walzl *et al.,* 2011

	Macrominerals (g/kgDM)						
	N	P	K	Ca	Mg	Na	Adapted from
Ryegrass	39.1	5.42	42.4	6.4	2.6	3.3	Wilman *et al.,* 1993
Plantain	36.5	4.5	43.8	20.1	2.4	7.2	
Ryegrass	25.1	3.8	29.9	5.8	2.1	2.2	Wilman *et al.,* 1994
Plantain	25.0	3.3	29.1	14.0	2.4	1.7	
Ryegrass	20.0	3.95	33.95	4.65	1.75	1.20	Pirhofer-Walzl *et al.,* 2011
Plantain	15.95	3.80	29.95	15.65	2.75	0.55	

Table 5: Micromineral content of plantain compared with ryegrass adapted from Hoskin *et al.,* 2006, Harrington *et al.,* 2006 and Pirhofer-Walzl *et al.*, 2011

	Co	Cu	Fe	Mo	Se	Mn	Zn	Cr	B	Adapted from
Ryegrass	0.35	9.3	1271	0.31	0.04	-	-	-	-	Hoskin *et al,* 2006
Plantain	0.36	14.0	795	0.34	0.06	-	-	-	-	
Ryegrass	0.19	7.90	151	0.64	0.02	99.0	22.0	-	19.0	Harrington *et al.,* 2006
Plantain	0.36	15.1	182	0.27	0.05	109	37.7	-	23.3	
Ryegrass	-	6.1	66.6	1.25	-	73.2	23.2	0.3	3.9	Pirhofer-Walzl *et al.,* 2011
Plantain	-	8.2	63.5	0.40	-	33.3	30.8	0.2	20.3	

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